Supplementary Appendix

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METHODS

PATIENTS, ETHICAL APPROVAL AND INFORMED CONSENT

Children aged 1 - 12 years were recruited at Queen Elizabeth Central Hospital (QECH), Blantyre Malawi. Post-mortem samples were from autopsies performed between January 2000 and May 2006 (**Supplemental table 1**) and all other patients were recruited between January 2008 and June 2011 (**Table 1**). The study was approved by the College of Medicine ethical board in Malawi (no. P.02/10/860) and by the Liverpool School of Tropical Medicine ethical board in the United Kingdom (no. 09.74). Informed consent was obtained from the parent or legal guardian of all the children admitted to the study and separate consent was sought for all post-mortem examinations.

PATIENT ENROLLMENT CRITERIA

1. Cerebral malaria (CM)
    a. Age between 1 and 12 years
    b. Blantyre Coma Score $\leq$ 2 on admission
    c. Peripheral *P. falciparum* parasitemia (any density)
    d. No improvement in Blantyre Coma Score (to a score above 2) after correction of hypoglycemia (if present)
    e. Informed consent from the parent or legal guardian

Retinal examination by an ophthalmologist then classified patients into retinopathy positive CM or retinopathy negative CM according to the presence or absence of features of the cerebral malaria retina as described previously[1].

2. Non-malarial coma

a. Age between 1 and 12 years

b. Blantyre Coma Score $\leq$ 2 on admission

c. Aparasitemic on peripheral blood film (thick)

d. No improvement in Blantyre Coma Score (to a score above 2) after correction of hypoglycemia (if present)

e. Informed consent from the parent or legal guardian

3. Uncomplicated malaria

a. Age between 1 and 12 years

b. Axillary temperature greater than 40°c

c. Blantyre coma score 5

d. Peripheral *P. falciparum* parasitemia (any density)

e. None of:

- Prostration (unable to sit unsupported)
- History of convulsions
- Jaundice
- Respiratory distress
- Hypoglycemia
- Severe anemia (Packed cell volume <15% or hemoglobin <5g/dL)

f. Informed consent from the parent or legal guardian

4. Mild aparasitemic febrile illness

a. Age between 1 and 12 years

b. Axillary temperature greater than 37.5°c

c. Blantyre coma score 5

d. Negative rapid diagnostic test (First Response; Premier Medical, India)

e. None of:

- Prostration (unable to sit unsupported)
- History of convulsions
- Jaundice
- Respiratory distress
- Hypoglycemia
- Severe anemia (Packed cell volume <15% or Hemoglobin <5g/dL)

f. Informed consent from the parent or legal guardian

5. Febrile controls having a lumbar puncture to exclude meningitis:

a. Age between 1 and 12 years

b. Axillary temperature greater than 37.5°c

c. Clinical decision to do a lumbar puncture to exclude meningitis (e.g. prolonged seizure, drowsy, high fever without focus)

d. Blantyre coma score 4 or greater

e. Negative rapid diagnostic test (First Response; Premier Medical, India)

f. Cerebro-spinal fluid not visually cloudy.

6. Healthy controls

a. Age between 1 and 12 years

b. Axillary temperature <37.5°c

c. Scheduled for elective surgical procedure under general anesthetic

d. No complicating medical condition (e.g. diabetes, inflammatory bowel disease, sickle cell disease)
e. Negative rapid diagnostic test (First Response; Premier Medical, India)
f. Informed consent from the parent or legal guardian

In all patient groups, children were excluded if they had clinical evidence of severe malnutrition (nutritional edema, severe wasting, dermatitis or hair changes)[2], had World Health Organization (WHO) clinical stage 4 HIV infection[3] or were on antiretroviral therapy. In the cerebral malaria and non-malarial coma group patients were excluded if they had microbiological evidence of bacteremia (pathogenic bacteria in culture media [Bactec; Becton Dickinson] after 3 days incubation; N=2) or if they had evidence of meningitis (>10 leukocytes/ mL or pathogenic bacteria on gram stain or after 3 days in culture; N=8).

Parasitemia was determined by thick and thin Field's stained smears and is expressed as asexual *P. falciparum* parasites per microliter based on individual patient full blood counts. Repeat smears were done 6 hourly on comatose children. None of the non-malaria coma cases with negative films on admission had subsequent positive smears. None of the patients in the control groups who had a negative rapid diagnostic test had a positive blood film for malaria.

POST MORTEM GROUP CHARACERISTICS AND HISTOLOGICAL STUDIES OF CEREBRAL MALARIA

Definitions for the two autopsy groups were as follows:

Cerebral malaria

a. Met WHO criteria for cerebral malaria[4] during life
b. Informed consent given for a post-mortem by parent or legal guardian
c. Malaria infected erythrocyte (IE) sequestration in cerebral vessels on histological assessment by medical pathologist

Non-Cerebral malaria controls

a. Met WHO criteria for cerebral malaria[4] during life
b. Informed consent given for a post-mortem by parent or legal guardian
c. Absence of IE sequestration on histological assessment by medical pathologist.

4 μm thick formalin-fixed paraffin-embedded samples were stained for fibrin accumulation using the martius-scarlet-blue (M.S.B.) trichromic staining method as described by Lendrum and colleagues[5] and for thrombomodulin and EPCR using the avidin- biotin complex method[6].

The degree of fibrin accumulation in cerebral microvessels was classified for each case in 50 vessels as greater than or equal to 50% of vessel containing fibrin, less than 50% of vessel containing fibrin or none. The intensity of immunostaining for EPCR and thrombomodulin was graded as absent, weak, moderate or strong based on reference micrographs of vessels of each staining intensity. The degree of IE sequestration was assessed by counting the number of parasites, as indicated by malaria pigment, in a 10x100 μm area of each of 50 vessels per patient. Each vessel

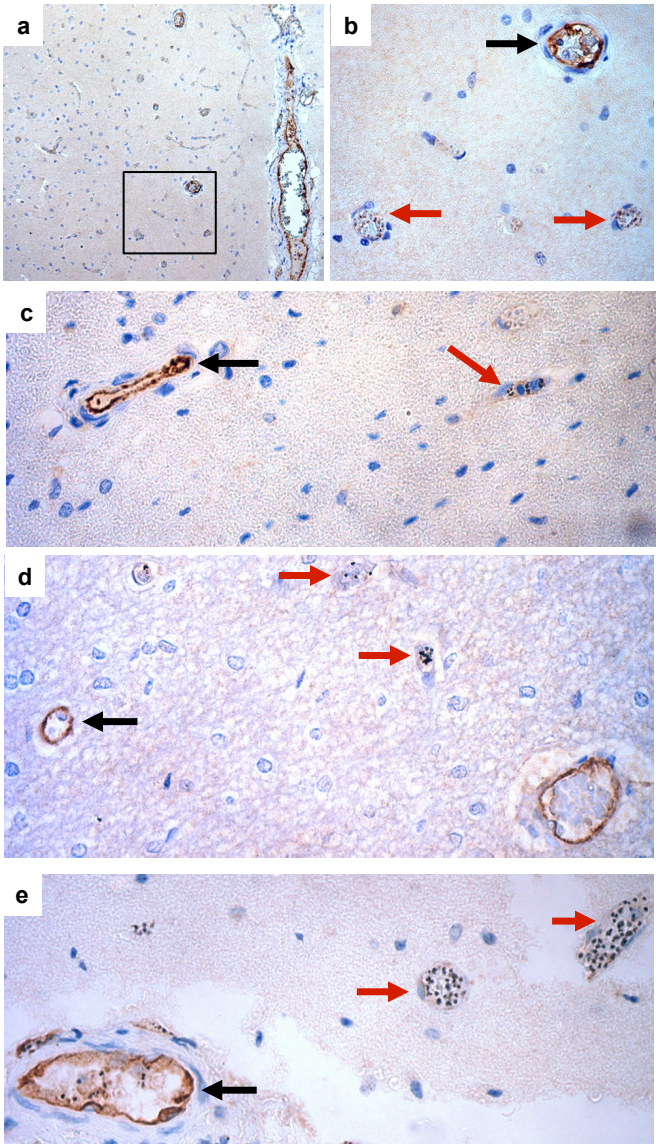
was scored as high [greater than 10 parasites], moderate [5 to10 parasites] or low [1 to 5 parasites]. Only good quality samples with minimal tissue damage were accepted for scoring: two cases and one control were excluded from scoring in brain tissue stained for EPCR and in subcutaneous tissue stained for thrombomodulin. The degree of fibrin accumulation and the staining intensity of EPCR were scored by one of the authors and by two independent medical pathologists, unaware of study aims and blinded to diagnosis. Each scorer scored 50 vessels per case and the results were combined, giving 150 vessels scored for each case for fibrin and 150 vessels scored per case for EPCR. Statistical analysis was done on the combined scores, with a variable included in the ordinal regression model to adjust for any variation between scorers. The percentages depicted in Figure 1 are means of the combined data from the three scorers. Micrographs were taken using a Leica DM1L microscope and a Micropublisher 3.3 RTV (QImaging) camera.

ASSAYS OF PLASMA AND CEREBRO-SPINAL FLUID ANTIGENS AND COAGULATION TESTS

Plasma and subcutaneous tissue samples were obtained from patients and controls between January 2008 and June 2011. Blood was obtained by venipuncture and citrate and benzamidine citrate plasma prepared as described previously[7]. Samples were frozen immediately at -80°C. Cerebro-spinal fluid (CSF) was obtained by lumbar puncture using aseptic technique and was spun (10 mins at 1200g) to remove any cells and then the supernatant frozen immediately at -80°C. Benzamidine plasma activated protein C (aPC) levels were measured by enzyme capture method as described elsewhere[8], the lower detection limit of the assay was 0.5 ng per mL. The upper limit for TAT was set at 156 µg/ L and for F1+2 at 8290 ng/ mL.

SUPPLEMENTAL FIGURES

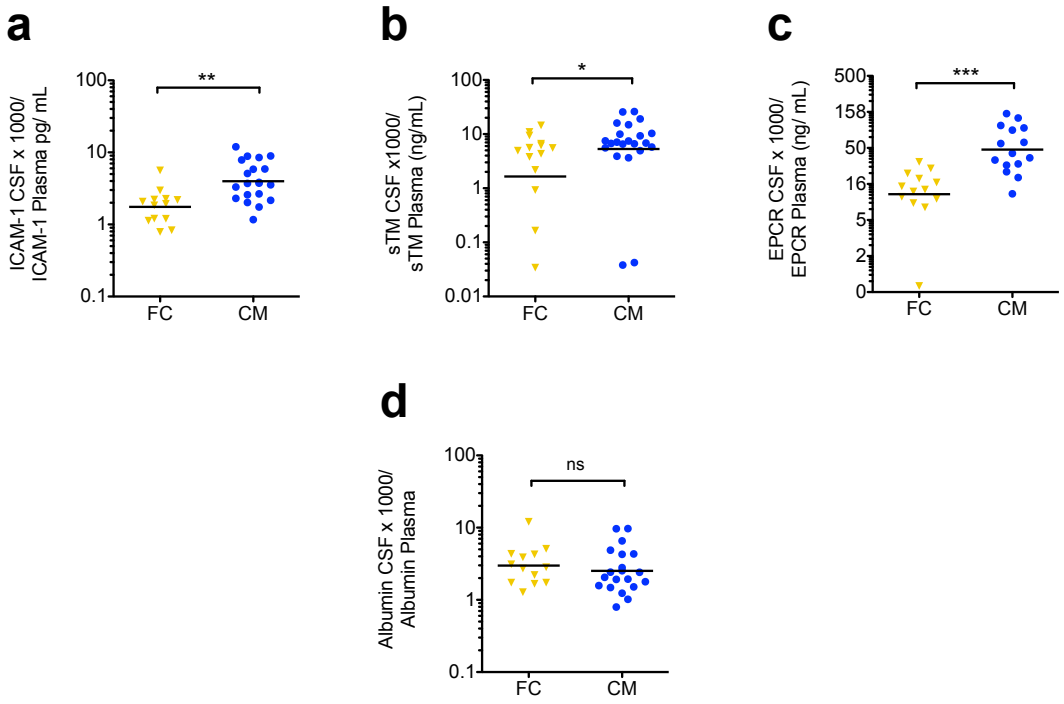
Supplemental Figure 1. Plasmodium falciparum-infected red blood cell sequestration in cerebral malaria is associated with low staining for endothelial protein C receptor



(b-e) Are medium power view images (x400) from brain tissue sections from four different fatal cerebral malaria cases. (a) is a low power view (x100) with (b) a detail of the area in the box. Black arrows indicate regions of moderate or strong staining for

endothelial protein C in vessels, or regions of vessels, with low IE sequestration, red arrows indicate vessels with low or absent staining for endothelial protein C in vessels containing high levels of IE sequestration as indicated by malaria pigment (black dots).

Supplemental Figure 2. Raised CSF-to-plasma ratios of soluble receptors and normal CSF-to-plasma ratio of albumin.



Scatterplots of CSF levels of (a) sICAM-1 (b) sTM and (c) sEPCR expressed as a ratio of paired levels in the plasma. (d) CSF-to-plasma ratio of albumin or albumin index (AI). Since albumin is not produced in the CSF or in the brain the AI gives an indication of the integrity of the blood-CSF barrier.

Supplementary Table 1: Post-mortem cases

Case (Diagnosis)	**Hemorrhagic lesions**[1]	**% vessels containing fibrin**[2]	**EPCR Brain**[3]	**TM SC.**[5]
Non CM 1 (Head trauma with subdural hematoma)	No	13	Moderate	-
Non CM 2 (*Streptococcal* pneumonia)	No	9	Moderate	Strong
Non CM 3 (Viral pneumonia)	No	13	Weak	Moderate
Non CM 4 (Viral pneumonia & Reye's syndrome)	No	2	Moderate	Moderate
Non CM 5 (Left ventricular failure)	No	7	Moderate	Moderate
Non CM 6 (Ruptured AVM[6] with subdural hematoma)	No	41	-	Strong
CM 1	Yes	93	Weak	-
CM 2	Yes	85	Weak	Weak
CM 3	Yes	87	Weak	Strong
CM 4	Yes	87	Weak	Moderate
CM 5	Yes	38	Weak	Strong
CM 6	Yes	76	-	-
CM 7	Yes	64	-	-
CM 8	No	85	-	Weak
CM 9	No	84	-	-
CM 10	No	65	-	-

[1]Denotes whether or not perivascular hemorrhages were seen at histological examination

[2]Denotes the overall percentage of vessels that contained fibrin staining using trichromic staining.

[3]Denotes the overall staining intensity for EPCR in post mortem brain sections by immunostaining as scored by two independent scorers blinded to diagnosis.

[4]Denotes the overall staining intensity for CD34 in post mortem brain sections by immunostaining as scored by two independent scorers blinded to diagnosis.

[5]Denotes the overall staining intensity for thrombomodulin (TM) in postmortem subcutaneous (SC) sections by immunostaining as scored by two independent scorers blinded to diagnosis.

[6]Cerebral arterio-venous malformation (AVM)

- Indicates that no suitable specimen was available for scoring due to limitations of samples and damage to small and delicate samples during antigen retrieval and staining.

Supplemental Table 2. Factors compared by age group

	Age 1-4 median (IQR)	N	Age 5-12 median (IQR)	N	P
TAT - µg/ L	34.9 (11.3-74.2)	47	17.6 (9.1-36.4)	19	0.1
aPC - ng/ ml	2.55 (1.65-3.48)	59	1.48 (0.99-2.65)	28	0.017
F1+2:aPC - ratio	1.72 (0.94-4.13)	58	2.03 (1.04-5.67)	26	0.57
PT - secs	16.65 (14.3-19.3)	42	16.8 (14.8-18.2)	16	0.92
aPTT - secs	34.9 (27.4-39.2)	42	30.1 (27.0-34.0)	16	0.06
TM - MFI	157.1 (85.3-178.2)	10	146.0 (90.9-301.8)	7	0.43
EPCR - % pos	8.7 (4.7-13.9)	10	7.42 (5.9-9.5)	7	0.7

TAT = thrombin anti-thrombin; aPC = activated protein; F1+2 = prothrombin fragment F1+2; PT = prothrombin time; aPTT = activated partial thromboplastin time; TM = thrombomodulin; EPCR = Endothelial protein C receptor; secs= seconds; MFI= mean fluorescence intensity; % pos= % positive events.

SUPPLEMENTAL REFERENCES

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